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TITLE: Three Dimensional Structure Determination of Botulinum Neurotoxin

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Aug. 1, 1994

Annual / Aug. 1, 1993- Jul 31, 1994

Three Dimensional Structure Determination of
Botulinum Neurotoxin

Contract #
DAMD 17-93-C-3118

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The immediate goals on structure and function relationship studies of botulinum neurotoxin are: 1) Determine the three-dimensional structures of botulinum neurotoxins isolated Heavy chain, Light chain and holo-neurotoxin at atomic resolution by x-ray crystallography. 2) Understand how the serologically distinct botulinum neurotoxins and isolated H- and L-chains are different in relation to their structure and function. 3) Based on the structure of the isolated chains and holo-neurotoxin, understand the toxins mechanism of action.

We have worked out the conditions to further purify serotype A (isoelectric focusing and control of aggregation), stabilize serotype A (addition of μM zinc acetate, proteolytic inhibitors, and ganglioside), and crystallize serotype A. We have also very carefully worked out condition to collect complete, accurate, and non-deteriorating (flash frozen -170°C) x-ray diffraction data. We have collected a large amount of both native (15 sets) and heavy atom derivative data (28 sets) that is used to phase the x-ray diffraction pattern to yield the 3-dimensional structure of the neurotoxin. As a feasibility study, we have initiated crystallization trials of serotype B and we have obtained small single crystals.

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x-ray crystallography, 3-D structure, botulinum neurotoxin

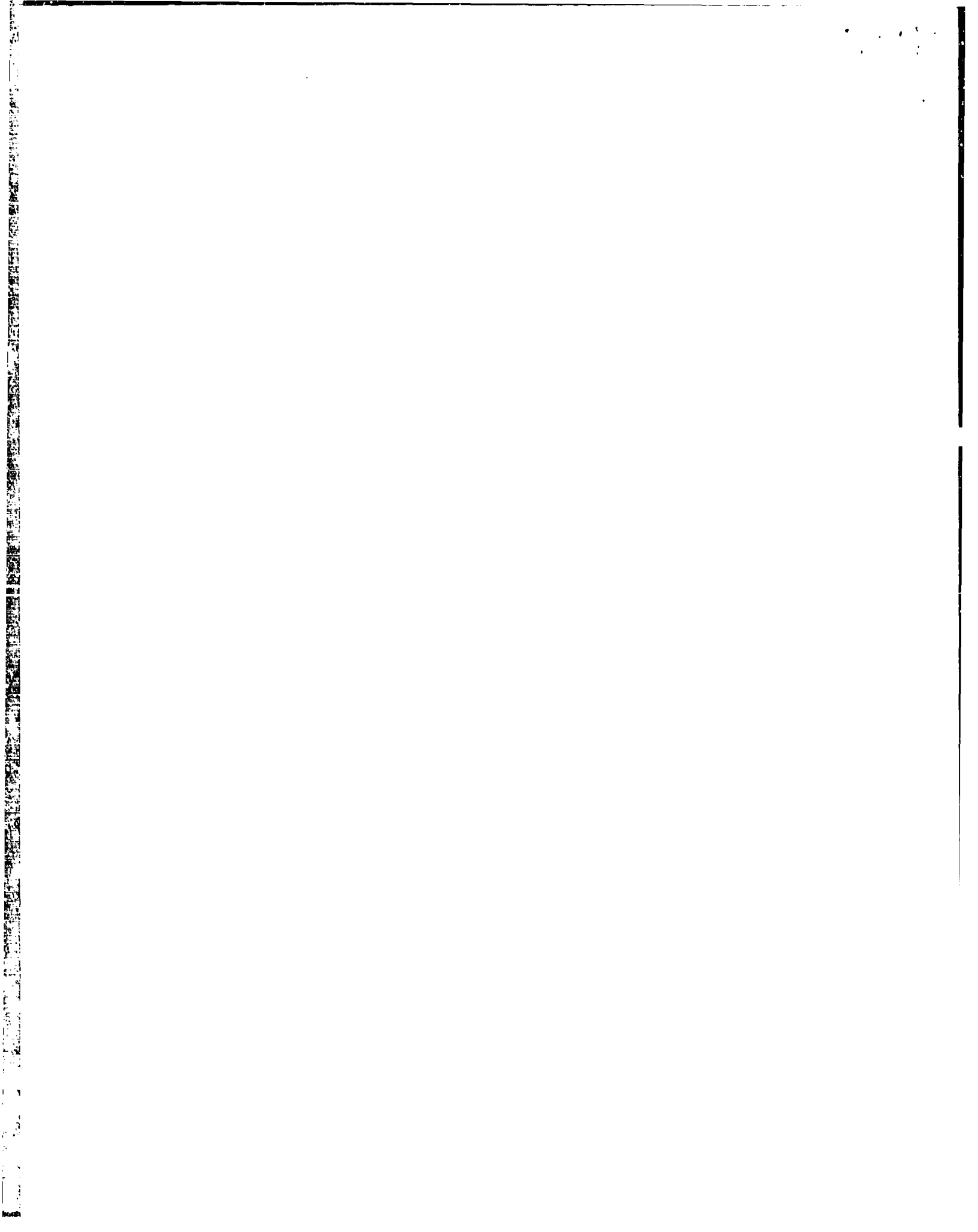
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FOREWORD

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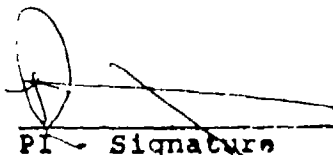
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Three-Dimensional Structure Determination of Botulinum Toxin
Contract: DAMD17-93-C-3118

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INTRODUCTION

Nature of Problem

To determine the 3-dimensional structure of botulinum neurotoxins and their isolated domains. The following specifications are listed in the contract section C - Statement of Work:

Crystallization of the 150 kDs holo-botulinum neurotoxins. Serotype A has been crystallized previously and crystallization conditions will be refined as necessary. Serotype B will be crystallized.

Determination of the 3-dimensional structure of serotype A. Heavy atom derivative screening is underway. Once 2 or 3 "acceptable" derivatives are known, data collection and processing of native and derivative data will be completed. The phases of the x-ray diffraction pattern will be phased, electron density maps calculated, and the structure will be determined.

Given the structure of serotype A, the technique of molecular replacement will be used to determine the structure of serotype B.

Crystallization of Heavy chain neurotoxin.

Determination of the 3-dimensional structure of the isolated heavy chain. Molecular replacement using the structure of the intact holo-neurotoxin if the structure has not changed substantially. If not, isomorphous replacement will be used.

Crystallization of Light chain neurotoxin.

Determination of the 3-dimensional structure of the isolated light chain. Molecular replacement using the structure of the intact holo-neurotoxin if the structure has not changed substantially. If not, isomorphous replacement will be used.

Similar studies on other serotypes.

Background of Previous Work

A factorial method developed in the P.I.'s laboratory was used to determine crystallization conditions of botulinum neurotoxin serotype A (Stevens et al, *J. Mol. Biol.* 222, 877 (1991)).

The most suitable crystal form of serotype A for X-ray analysis are bipyramidal shaped crystals that crystallize in the hexagonal space group P321 (or P3221) with one dimer per asymmetric unit. The unit cell dimensions are $a = b = 170.5 \text{ \AA}$, $c = 161.7 \text{ \AA}$. The crystals are composed of approximately 50% solvent/50% protein and diffract to 3 \AA resolution (Stevens et al, *J. Mol. Biol.* 222, 877 (1991)).

Native and derivative data sets to 3.0 Å resolution have been collected on serotype A in house (Room 405 Stanley Hall) at the University of California, Berkeley using a shared (with 3 other research groups) Rigaku RU-200 rotating anode generator and R-axis Image Plate area detector system.

All work described in this proposal have been conducted by contract personnel (1 postdoc and 1 grad student) and startup funds obtained from the UC-Berkeley Chemistry Department (1 postdoc and 2 grad students).

Purpose of Present Work

To continue the contracted work on the 3-dimensional structure determination of botulinum neurotoxin and understand the toxins structure, function, and mechanism of action. Specifically we will locate the heavy atom positions of data collected and attempt to phase the diffraction pattern to allow us to trace the electron density of the protein molecule. We will also need to collect more x-ray data as we continue to improve our model of the protein structure.

Methods of Approach

Using the technique of multiple isomorphous replacement, the 3-dimensional structure of botulinum neurotoxin will be solved using diffraction data from native and heavy atom derivative data sets that are collected in house or at synchrotron facilities. The technique requires one to:

- 1) Bind heavy atoms to derivitize the protein crystal and locate the position of the heavy atom using Patterson methods. The derivitized protein crystals must be isomorphous with the native protein crystals (except for the heavy atom itself) for the information to be interpretable.
- 2) Once the position of the heavy atoms have been located, approximate phases can be calculated to allow one to observe the electron density of the protein molecule. The program HEAVY (Terwilliger, 1981) and the CCP4 program suite (CCP4, 1979, The SERC (UK) Collaborative Computing Project No. 4, a Suite of Programs for Protein Crystallography, distributed from Daresbury Laboratory, Warrington, WA4 4AD, UK) will be used to combine and phase heavy atom derivative data. All computations will be conducted on a Silicon Graphics INDIGO graphics workstation.
- 3) Fitting of electron density will be made on a Silicon Graphics INDIGO graphics workstation using the program TOM and O (version 5; Alwyn Jones, 1990).
- 4) Upon completion of fitting the electron density, the experimental model will be refined using the program XPLOR (version 3.0; Brunger, 1992) installed on a Silicon Graphics INDIGO graphics workstation.

An alternative to isomorphous replacement is the method of molecular replacement which depends on the presence of related structures in different crystals. Proteins which are homologous and have closely similar structures are particularly useful. The near identity of the structures implies relations between different structure amplitudes and phases which are helpful in solving phase problems. Serotypes A, B and E are similar as well as dissimilar (pharmacologically similar, antigenically different). Hence, once a structure is obtained for one serotype, the analysis of crystals of other serotypes can be aided by the molecular replacement technique. This is based on the assumption that the overall tertiary structure of all three serotypes are similar. If this is not the case, then a search for heavy atom derivatives will have to be conducted for all three serotypes.

BODY

Experimental Methods

At the beginning of the contracted work, x-ray diffraction quality crystals had been obtained that diffracted to 3.0 Å resolution (Stevens et al, *J. Mol. Biol.* **222**, 877 (1991)). During the past year we have obtained 101.89 milligrams of purified botulinum neurotoxin serotype A (see Appendix A1). All of the neurotoxin has been used in the studies listed. Below is a list of experiments that have been conducted in the past year:

1) Data have been collected on 15 native data sets from 3 different x-ray sources (see Table I). A number of native data sets have been collected because of isomorphous problems between native data sets, data sets collected at different temperatures to determine the most stable method to freeze protein samples, and the collection of data at different locations. Because the native data set is so important in phasing with the heavy atom data set, it is critical to collect numerous native data sets to ensure isomorphism. We have traveled to the Stanford Synchrotron Research Laboratory in Palo Alto, CA and the Photon Factory in Tsukuba, Japan to collect synchrotron data. All other data have been collected in house on conventional rotating anode x-ray sources and R-axis image plate system. Merging statistics in Table I include all data to the highest resolution bin without throwing away any data.

2) Heavy atom derivative data sets have been collected on 28 different heavy atom derivative solutions from 3 different x-ray sources (see Table I). The large number of heavy atom data sets have been collected because the heavy atom solution conditions have varied with time of exposure to heavy atom solution, concentration of heavy atom solution, addition of salts and inhibitors to alter the binding activity of heavy atom solutions. Temperature of data collection has also been changed as well as the x-ray source. Merging statistics in Table I include all data to the highest resolution bin without throwing away any data.

3) Interpretation of Patterson maps to locate the positions of the heavy atoms is currently underway on the data sets listed in Table I.

4) Flash Freezing of Protein Crystals.

A technique that allows us to flash freeze the protein crystals in liquid nitrogen (-175°C) has been worked out. By freezing the protein crystals in liquid nitrogen the protein sample is stable indefinitely in the x-ray beam. If the protein crystals are not flash frozen, the crystal lifetime in the x-ray beam deteriorates within hours. The diffraction pattern deteriorates causing a decrease in resolution of atomic structure. This effect is caused by both the heating of the crystal sample as it is exposed to x-rays and the radiation which produces free radicals in the protein molecules. Since an average data set requires approximately 24 hours of continuous x-ray exposure, flash freezing the protein crystals is necessary for the collection of complete and accurate data that is not affected by deterioration of crystal sample. Radiation damage is particularly harmful to the protein

crystals when using high energy synchrotron radiation and thus, flash freezing is even more important.

Conditions for flash freezing are as follows - Protein crystals grown in the crystallization buffer (2 M NaFormate, 100 mM Hepes pH 8.5) are washed in 3 series of crystallization buffer + cryoprotectant. The serial washes are necessary to remove any water molecules located on the surface of the protein crystal and to provide a cryoprotectant for the protein crystal. Once the protein crystal has been washed, it is placed immediately in a stream of liquid nitrogen that is stable at -175°C.

5) Because of problems with reproducibility in quality of large (0.3mm x 0.3mm x 0.3mm) single crystal growth, 2 factors were investigated -

Protein purity - Although holo-toxin shows a single clean band (150 kD) on non-denaturing gel and two single clean bands (100 kD + 50 kD) on denaturing gels, isoelectric focusing gels show that the holo-toxin obtained from the Univ. of Wisconsin is composed of 3 isoforms (pH 6.5, 6.3, and 6.1) plus a few smaller fractions. Since protein crystal growth is based on the packing of identical molecules in a crystal lattice, pure material is necessary. Using a BioRAD Rotaphor, we have been able to successfully isolate the different isoforms. We have been able to crystallize these isoforms although we do not have enough material at present to aggressively pursue this path since the additional purification step reduces the yield of usable protein by 33%. The most likely explanation of this observation is that nicking of the toxin between the heavy and light chains may vary from molecule to molecule.

Protein aggregation - It has been observed that protein that is either concentrated at levels higher than 10 milligrams/milliliter or protein solutions that have been sitting around in solution for more than a few days after the final column purification step do not yield large single crystals suitable for x-ray diffraction. Instead, numerous small crystals are obtained that are too small even for synchrotron radiation experiments. In order to circumvent this problem we have specifically instructed the DasGupta laboratory to not concentrate the protein solution beyond 10 mg/ml. We also use the protein immediately upon receiving the protein samples from the DasGupta laboratory. This makes the crystallization of the protein difficult since crystallization conditions vary from batch to batch (this is normal based on previous experience on other proteins). If the protein is not used immediately, further purification is required in house. The most likely explanation of this observation is that protein aggregation is occurring.

6) Crystallization of serotype A with ganglioside GT1b. Similar to the above arguments for stabilizing the neurotoxin structure with inhibitors to the active proteolysis site, one can stabilize the ganglioside binding site by co-crystallizing the neurotoxin with ganglioside. Using GT1b ganglioside (purchased from IsoSep in Sweden), we have co-crystallized the neurotoxin with the ganglioside and collected x-ray diffraction data. This information will aid us in determining the ganglioside binding site on the neurotoxin surface once the structure is known and allow us to understand how the neurotoxin recognizes and binds to the neuronal surface.

7) Crystallization of botulinum neurotoxin serotype B.

We have obtained 3 milligrams of fully nicked BT serotype B from Dr. Eric Johnson at the Food Research Institute, University of Wisconsin, Madison, WI. Using the incomplete factorial method for the crystallization of protein molecules, we obtained small single crystals under conditions that were similar to serotype A. The diffraction quality of the crystals was poor but this is most likely due to the age of the protein (Dr. Johnson informed us that the protein was several months old) and the size of the protein crystals. This work did not require a great deal of time on our behalf since Dr. Eric Johnson provided a small amount of purified protein and we immediately set up crystallization trials with all of the material in a few days. This work therefore did not detract from the serotype A work. If further work is to be pursued, another researcher will be required in order to complete the study.

This work completes goal C2 in the contracted work.

8) Determination of kinetic parameters of proteolysis by serotype A.

Because of the need for a means to evaluate the quality of protein used in crystal growth experiments in addition to the fact that we need to learn as much as possible about the behavior of the protein during crystal growth experiments and to help us understand the function of the neurotoxin once the structure is determined, we have initiated simple kinetic assays to evaluate the neurotoxins proteolytic activity. The substrate being used is a 17 amino acid peptide of the synaptic vesicle protein SNAP-25. After several months of attempting to optimize proteolytic activity, it was determined that the zinc concentration of the toxin must be 100 micromolar. If the concentration of any zinc salt is above the micromolar range, the neurotoxin activity is inhibited. If the concentration of any zinc salt is below the micromolar range, the neurotoxin activity is very very low (less than 10% of full activity). With this information, we now routinely use 100 μ M zinc acetate in all crystallization conditions to help stabilize the neurotoxin. It cannot be overemphasized that micromolar concentrations of zinc are absolutely necessary for full activity. In similar zinc protease structures, it is observed that correct concentrations of zinc atoms can possess either structural/catalytic or both roles in aiding the proteolytic activity. In the case of botulinum neurotoxin, we believe zinc to play both roles since we have observed that zinc salts stabilize the crystal growth under the optimal conditions (micromolar).

Analogous to other crystal structure determinations, it has frequently been observed that inhibitors for enzymes aid in the stabilization of protein molecules by locking the enzyme into a single stable conformation. By using the above kinetic assay, we have been able to screen potential inhibitors of the neurotoxin to stabilize a single conformation. The inhibitors being investigated including peptide-like analogs synthesized by Professor Paul Bartlett in the Dept. of Chemistry of UC-Berkeley. Based on the protease recognition site, we are investigating the inhibitors Z-Ala-Gly-P-Phe-, Z-Phe-P-Leu-Ala-, Z-Gly-Ala-P-, and Cbz-Gly-P-Leu-Gly-. The "-P-" moiety is a phosphate backbone in place of the amide backbone that strengthens the peptide bond and does not allow cleavage by the protease. The inhibitor does however have the recognition elements that the neurotoxin binds.

All work on this aspect of the project has been conducted by personnel supported by the Department of Chemistry at UC-Berkeley. Because the amounts of neurotoxin needed to conduct kinetic assays is so small, it did not inhibit the structural studies in any manner.

9) Recombinant DNA work on serotype A domains to produce protein for crystallography experiments.

The present crystals of botulinum neurotoxin diffract to 3.0 Å resolution. At this resolution, we will be able to determine the 3-dimensional structure of the neurotoxin and observe secondary structure elements (alpha helices, beta strands, and beta sheets). It will be difficult to observe detailed side chain interactions of the smaller side chains (the larger side chains should easily be observable). In order to obtain a more detailed picture of the protein structure, recombinant DNA work on isolated domains (binding, translocation, catalytic) have been initiated with the goal of crystallizing the domains and determining the 3-D structure by x-ray crystallography. The approach of "divide & conquer" has been used in numerous examples to determine the structure of regions of protein molecules (i.e. SH2 & SH3 domains of tyrosine kinases). To date, in collaboration with Dr. Jim Marks at UCSF, we have cloned and expressed each of the domains and are presently working on increasing the expression levels in *E. coli* with various expression systems. Presently, only the binding domain is expressing at levels high enough to clearly see on coomassie or silver stained gels. Although this aspect of the work is probably the most difficult, it also has a high payoff if successful. Furthermore, we have obtained preliminary success on the binding domain that suggest we can accomplish the task.

The concern of correctly folded conformation of the isolated domains has been examined by ELISA assays performed by the Marks laboratory at UCSF and the binding domain appears to be folded in its native conformation. We also have assays in house (proteolytic assay) to confirm the conformation of the catalytic domain and inhibition assays on certain neurotoxin sensitive cell lines (PC12) could be used to confirm structure and activity.

This work is required to understand the structure/function of the neurotoxin since it's mechanism of action requires the separation of each domain at some point during the neurotoxins activity on neuronal cells.

All work on this aspect of the project has been conducted by personnel supported by the Department of Chemistry at UC-Berkeley. Proper authorization to conduct recombinant DNA work on fragments of botulinum neurotoxin were obtained from the Biosafety Officer, Office of Environment, Health and Safety (see Appendix B).

This work is in line with goals C5 and C7 of the contracted work, to crystallize the isolated domains of botulinum neurotoxin. Large quantities of purified protein is required and a recombinant approach is being taken. The alternative approach is by purifying the light and heavy chains from one another. This approach would require twice or greater the amount of holo-neurotoxin plus purification. Secondly, it is impossible to separate the binding domain from the translocation domain in this fashion.

Relationship to Goals of Research

All work to date focuses on the single goal of determining the 3-D structure. Although numerous side projects have emerged (kinetic assay, recombinant DNA work ganglioside binding), all of these projects have greatly aided the crystallization and structure determination goal. Furthermore, the information obtained during these studies will be mandatory in understanding the function of the neurotoxin once the structure is known.

CONCLUSION

We have worked out the conditions to further purify the protein (isoelectric focusing and aggregation), stabilize the protein (zinc acetate, proteolytic inhibitors, ganglioside), and crystallize the protein. We have also very carefully worked out condition to collect complete, accurate, and non-deteriorating x-ray diffraction data. We have also collected a large amount of both native (7 sets) and heavy atom derivative data (20 sets) that is used to phase the x-ray diffraction pattern to yield the 3-dimensional structure of the neurotoxin. As a feasibility study, we have initiated crystallization trials of serotype B and we have obtained small single crystals (completion of Contract Goal C2).

Based on the previous years work, the contracted work is on schedule. Perhaps the only weak point in the present investigations is a lack of manpower since all personnel conducting non-contracted work will expire on Sept. 1st. These individuals have proven priceless in aiding in the improvements concerning proteins stability and crystallization.

X-ray Data Collected Between August 1, 1993-July 31, 1994
on Botulinum Neurotoxin Serotype A (Crystal Space group P3₁2₁)

| Data Set Name | Derivative | X-ray Source | Resolution Å | Unit Cell Å | #Reflections Collected | #Reflections Unique | Rmerge % |
|---------------|-----------------------|--------------|--------------|-------------|------------------------|---------------------|----------|
| BT118 | native | Raxis | 4.0Å | 165.235 | 53,021 | 20,090 | 16.3 |
| BT126HG | mercurous nitrate | Raxis | 3.5Å | 160.03 | 64,447 | 26,534 | 13.7 |
| BT14 | native | Raxis | 4.0Å | 164.97 | 78,606 | 21,959 | 18.0 |
| BT245 | native | SSRL | 3.6Å | 164.5 | 119,541 | 28,970 | 9.0 |
| BT910 | native | Raxis | 5.0Å | 167.42 | 15,255 | 5,817 | 8.3 |
| BT A10 | native | SSRL | 3.3Å | 169.77 | 138,823 | 33,715 | 13 |
| BT A9 | native | SSRL | 3.3Å | 169.77 | 115,541 | 27,870 | 12 |
| BTEMP | emp(Hg) | SSRL | 3.8Å | 166.73 | 81,788 | 24,239 | 13.5 |
| BTHG1 | mercurous iodide | SSRL | 4.0Å | 165.28 | 27,876 | 11,331 | 11.2 |
| BT PB | lead nitrate | SSRL | 3.3Å | 166.045 | 78,414 | 21,352 | 7.67 |
| BT PB531 | lead nitrate | Raxis | 4.5Å | 166.2 | 159.98 | 13(1) | 10 |
| BTSS13 | native | SSRL | 3.8Å | 164.45 | 59,909 | 19,031 | 10 |
| BTSS154 | NATIVE | SSRL | 3.5Å | 167.01 | 76,000 | 17,595 | 10 |
| BTSS054 | native | SSRL | 3.5Å | 165.0 | 74,242 | 20,568 | 9.0 |
| BTSSRL2 | native | SSRL | 3.3Å | 165.14 | 152,373 | 36,892 | 13.5 |
| BTTL5415 | 5mm thallium nitrate | Raxis | 4.0Å | 165.11 | 159.74 | 16 | 16 |
| BTTL1414 | 1mm thallium nitrate | Raxis | 4.0Å | 164.94 | 160.07 | 11 | 11 |
| BTHGAU425 | mercury silver iodide | Raxis | 5.0Å | 164.37 | 159.04 | 20(1) | 20(1) |
| BT PB*24H | lead nitrate (24h) | Raxis | 5.0Å | 164.8 | 159.93 | | |
| BT PB1mm | lead nitrate | Raxis | 5.0Å | 166.0 | 159 | | 17(1) |
| BT PB0232 | 2mm lead nitrate | Raxis | 5.0Å | 167.02 | 160.01 | | 19(1) |
| BTAC3 | mercurous acetate | PF | 3.5Å | | | | |

R-axis - Rigaku RU-200 Generator with R-axis Image Plate System at UC-Berkeley - 1.56Å
SSRL - Stanford Synchrotron Research Laboratory with Mar Image Plate System - 1.0Å
PF - Photon Factory with Fuji Image Plates at Tsukuba, Japan - 1.0Å
(I) - incomplete

X-ray Data Collected Between August 1, 1993-July 31, 1994
on Botulinum Neurotoxin Serotype A (Crystal Space group P3₁2₁)

| Data Set Name | Derivative | X-ray Source | Resolution Å | Unit Cell Å | #Reflections Collected | #Reflections Unique | Rmerge % |
|---------------|------------------------|--------------|-----------------|----------------|---------------------------|------------------------|-------------|
| BTHGN | mercurous nitrate | SSRL | 3.5Å | | | | |
| BTME2 | mercury chloride 1mm | SSRL | 4.0Å | | | | |
| BTNATIVE | native | SSRL | 3.5Å | | | | |
| BTSD1 | sialic acid(Hg) | SSRL | 3.7Å | | | | |
| BTSD7 | sialic acid (Hg) | SSRL | 3.5Å | | | | |
| BTTLN | thallium nitrate | SSRL | | | | | |
| BTCD05321 | 0.5mm cadmium chloride | Raxis | 4.0Å | 165.03 | 160.99 | | 11.7 |
| BTGH423 | mercurous iodide | Raxis | | 164.4 | 157.6 | | |
| BTOSRED | iridium chloride | Raxis | 6.0Å | 165.4 | 158.96 | | |
| BTU5418 | uranyl acetate | Raxis | 4.0Å | 162.98 | 160.93 | 11,782 | 16.7(1) |
| BTNAT55 | native | Raxis | 4.0Å | 165.5 | 159.93 | 9,265 | 16(1) |
| BT0USAC | mercurous acetate | Raxis | 3.0Å | 162.247 | 160.406 | 24,394 | 13 |
| BT2MBTL | 2mm mercuribenzoate | | | | | | |
| | 1mm thallium nitrate | Raxis | 3.4Å | 161.28 | 159.6 | 15,302 | 15 |
| BT2PHGH515 | diphenyl mercury | Raxis | 3.8Å | 165.72 | 159.7 | 12,824 | 13 |
| NAT1B | native | PF | | | | | |
| TN1B | thallium nitrate | PF | | | | | |
| BT223PB | lead nitrate | Raxis | 5.0Å | 165.78 | 158.76 | | 17(1) |
| BT410M135 | mercuribenzoate | Raxis | 3.8Å | 161.64 | 159.62 | 19,270 | 16.2 |
| BT49MB5 | mercuribenzoate | Raxis | 3.8Å | 161.64 | 159.62 | 14,325 | 12 |
| BTPB1311 | lead nitrate | Raxis | 5.0Å | 163.25 | 160.44 | | 13(1) |
| BTCD | cadmium chloride | Raxis | 4.0Å | 162.97 | 160.22 | 9,845 | 10.6 |
| BTMERS | methyl acid (Hg) | Raxis | 3.8Å | 160.8 | 160.2 | 11,198 | 13 |

R-axis - Rigaku RU-200 Generator with R-axis Image Plate System at UC-Berkeley - 1.56Å

SSRL - Stanford Synchrotron Research Laboratory with Mar Image Plate System - 1.0Å

PF - Photon Factory with Fuji Image Plates at Tsukuba, Japan - 1.0Å

(1) - incomplete

APPENDIX

| | |
|--|----|
| Reporting Letter from SubContractor Univ. Wisconsin (B.R. DasGupta) | A1 |
| Univ. California-Berkeley Recombinant DNA Approval Document | B1 |
| Updated Emergency Response Plan | C1 |

FOOD RESEARCH INSTITUTE

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Annual report on the subcontract from DOD (DAMD 17-93-C-3118)

Michael W. Pariza
Director, Chair
Professor
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To: R. C. Stevens, University of California, Berkeley

Fun Sun Chu
Professor
263-6932

From: B. R. DasGupta *BRD*

Date: August 23, 1994

Dean O. Cliver
Professor
263-6937

Between October 28, 1993 and August 1, 1994, 101.8 mg of pure type A neurotoxin was sent to Prof. Ray Stevens to prepare crystals (see table below).

Eric A. Johnson
Associate Professor
263-7944

Charles W. Kaspar
Assistant Professor
263-6936

John B. Luchansky
Assistant Professor
263-7280

Amy C. Lee Wong
Assistant Professor
263-1168

Robert K. Bush
Professor
(also Medical School)
262-7056

E. M. Foster
Former Director
262-4696

John K. McAnelly
Senior Scientist
Research Liaison
263-6826

Type A neurotoxin was produced and purified according to a published procedure (DasGupta and Sathyamoorthy, Toxicon 22, 415, 1984). To obtain a higher degree of purity than achieved before (Toxicon 22, 415, 1984) the procedure was modified in the following two manners: i) In the reported step 4 chromatographic fractions with an A_{260}/A_{278} ratio of no more than 0.55 across the first peak were pooled (rather than up to 0.6, and higher than 0.6, as was reported before); this decreased the yield, but we think gives purer neurotoxin. ii) After step 6, the recovered neurotoxin pool was enriched with NaCl (0.3 M), then incubated with para amino-isopropyl-thiogalactoside-agarose beads (which selectively binds the hemagglutinin proteins that often remain present with the neurotoxin after DEAE-Sephadex chromatography), filtered and precipitated with ammonium sulfate prior to Step 7, which is chromatography on SP-Sephadex C-50 column.

The purified neurotoxin stored as precipitate (ammonium sulfate) was recovered by centrifugation and dissolved in 10 mM HEPES buffer, pH 7.0, containing 0.1 M KCl and 0.002 M Na-azide. The solution was centrifuged to remove insoluble material. The supernatant was concentrated by centrifugation using Amicon centricon 100 to a small volume (~0.5 ml). To the solution remaining on top of the filter 1.0 ml buffer was added and centrifugation was repeated for concentration. After repeating this step three times the protein concentration was determined based on absorbance and the protein was diluted to a desired concentration, e.g., ~18.0 mg/ml or 10 mg/ml. This solution was packed in ice and shipped to Berkeley by overnight express delivery service as tabulated.

Bibhuti R. DasGupta
Senior Scientist
263-6806

| | Date | ~mg/ml | ~mg shipped | Batch # |
|-----|----------|--------|-------------|-------------|
| 1. | 10/28/93 | 17.90 | 11.60 | 65 10/28/93 |
| 2. | 12/8/93 | 16.47 | 6.60 | 65 12/8/93 |
| 3. | 2/2/94 | 25.77 | 3.35 | 66 2/2/94 |
| 4. | 2/16/94 | 10.0 | 14.2 | 66 2/9/94 |
| 5. | 2/25/94 | 12.4 | 8.68 | 67 3/23/94 |
| 6. | 4/14/94 | 10.0 | 8.59 | 67 4/14/94 |
| 7. | 4/28/94 | 10.0 | 10.60 | 67 4/27/94 |
| 8. | 5/16/94 | 10.0 | 13.50 | 68 5/13/94 |
| 9. | 5/25/94 | 10.0 | 15.40 | 68 5/23/94 |
| 10. | 8/1/94 | 10.0 | 9.37 | 69 7/31/94 |

Total = 101.89 mg

October 29, 1993

Professor Ray Stevens
College of Chemistry

RE: Review of Recombinant DNA Protocol

Your application for working with recombinant DNA of Botulism Neurotoxin has been reviewed. Because you are using only partial fragments of the toxin protein and the documentation of non-toxicity of these fragments, it was determined that your work is exempt from NIH Guidelines under Section III-D and Appendix C-II. It does not require a campus Biohazard Use Authorization.

If you make any changes to your experimental protocol, you must notify the Committee on Laboratory and Environmental Biosafety and have the additional work reviewed.

If you have any questions, please call me at 643-6562.



Chris Carlson
Biosafety Officer

CRČ/crc

cc: Mike Chamberlin, Committee on Laboratory & Environmental Biosafety
Harry Chiladakis, College of Chemistry
Susan Spencer, EH&S

"Exempt" rDNA Registration Form
Office of Environmental Health and Safety
Laboratory & Environmental Biosafety Committee

Principal Investigator: Raymond C. Stevens Telephone: 643-8285

Date: August 6, 1993 Department: Chemistry *Volume: 5 (liters)

Microorganisms Containing Recombinant DNA Molecules

Only NIH "exempt" classes of host-vector systems may be performed without prior Laboratory & Environmental Biosafety Committee written approval. You must complete all three parts of this form to verify that your research is "exempt" from the NIH guidelines. If the recombinant organism(s) generated in your research meet the "exempt" criteria presented in this form, verify this classification by *initialing* where appropriate for the host-vector system(s) employed and certifying that the subject recombinant organism(s) are not represented among any of the "non-exempt" exceptions presented in this form.

Part One: Details on the Host-Vector System:

1. Source(s) of DNA Gram positive prokaryote: Clostridium botulinum
2. Nature of the inserted DNA sequence(s) fragments (~1300 bp, ~2500 bp, ~2000 bp) of a gene that encodes a protein with three distinct structural domains: Light chain, Nd terminus of the heavy chain
3. Host(s) and Vector(s) to be used Host: E. coli K-12 strains: TG1, JM101 or DH5. Vector: nonconjugative plasmid, c.d. ColEI
4. Will a deliberate attempt be made to obtain expression of a foreign gene, and if so, what protein(s) will be produced? 1) light chain catalytic domain; 2) N-term of heavy chain: translocation domain; 3) C-term of heavy chain: binding domain.
5. List any unusual characteristics of this organism or its gene products:
The organism is classified as BL-2. The distinct and separate portions of the protein: B. neurotoxin has LD₅₀=0 for vertebrates. The lab proposes work entailing expression of these separate domains and not of the intact protein.

*All rDNA research which generate cultures exceeding 10 liters in volume must be reviewed and authorized by the Laboratory & Environmental Biosafety Committee or a delegated representative thereof, in accordance with the NIH "Guidelines for Research Involving rDNA Molecules" (1987).

(Please complete reverse side of this form)

A. *E. coli* K-12 Host-Vector Systems.

RS I certify that the host organism is an *E. coli* K-12 laboratory strain; and

- i. RS the *E. coli* host does not contain conjugation proficient plasmids or generalized transducing phages; and/or
- ii. RS lamda or lamdoid or F1 bacteriophages or nonconjugative plasmids have been used as vectors.

** (Note: Experiments involving the insertion into *E. coli* K-12 of DNA from prokaryotes that exchange genetic information with *E. coli* may be performed with any *E. coli* K-12 vector (eg. conjugative plasmid). When a nonconjugative vector is used, the *E. coli* K-12 host may contain conjugation proficient plasmids (either autonomous or integrated) or generalized transducing phages).

B. *Saccharomyces* Host-Vector Systems:

— I certify that this recombinant is contained in either a *Saccharomyces cerevisiae* or *Saccharomyces uvarum* host vector system.

C. *Bacillus subtilis* Host Vector Systems:

— I certify that this recombinant is contained in an asporogenic *Bacillus subtilis* strain which does not revert to a sporeformer with a frequency greater than 10^{-7} .

Part Three: Exceptions to the "Exempt" Classification:

Research involving recombinant organisms meeting any of the following criteria are not considered "exempt" and may not be conducted without authorization from the campus Laboratory and Environmental Biosafety Committee. Read each of the four "non-exempt" criteria below. If these exceptions do not apply to your work, indicate this by initialing the statement below.

1. Experiments involving DNA from Class 3, 4 or 5 organisms or from cells known to be infected with these agents.
2. Experiments involving the deliberate cloning of genes coding for the biosynthesis of molecules toxic for vertebrates.
3. Deliberate transfer of a drug resistant trait to microorganisms that are not known to acquire it naturally if such acquisition could compromise the use of the drug to control disease agents in human or veterinary medicine or agriculture.
4. Deliberate release into the environment of any organism containing recombinant DNA.

RS I certify that I have read and understand the above descriptions of "non-exempt" recombinant experiments and believe to the best of my knowledge that the subject organism(s) of this registration does not meet any of these "non-exempt" criteria.

I certify that the above organism(s) described in this form do not pose a unique hazard and require no special precautions other than "good microbiological practices". I understand that it is my responsibility under the NIH Guidelines for rDNA Molecules and the Campus Biohazard and Carcinogen Safety Manual to adhere to all practices of biosafety contained in these documents.

Ray St...
Principal Investigator

9/1/93
Date

REGISTRATION OF PROJECTS* INVOLVING RECOMBINANT DNA (rDNA)

Principal Investigator: Raymond C. Stevens

Date: August 18, 1993

Department or Research Unit: Chemistry

Campus Telephone: 3-8285

Mailing Address: Department of Chemistry
University of California
Berkeley, CA 94720

Registration #:

I. Projects Exempt from the NIH Guidelines: (if more than one project, please label accordingly)

1. Nature of project: cloning and expression of gene fragments encoding C. Botulinum structural domains.
2. Section of the NIH Guidelines relevant to exemption: III-0

II. Projects Covered by the NIH Guidelines Using E. coli K-12 Host-Vector Systems:

1. Nature of project (identify source of DNA to be cloned and describe the nature of the inserted DNA sequences): Source: Gram positive prokaryote: Clostridium botulinum. Nature: gene fragments encoding structural domains.

Check all items that are applicable:

- () project involves the insertion into E. coli K-12 of DNA from prokaryotes that exchange genetic information but are not on the "list of exchangers" in Appendix A of the NIH Guidelines.
- () project involves the deliberate attempt to have E. coli K-12 efficiently express any gene coding for a eukaryotic protein.

2. Host-vector system:

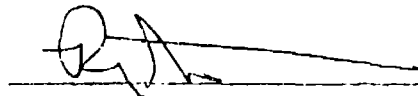
vector(s): nonconjugative plasmid, e.g., ColE1.

host(s): E. coli K12 strain: JM101

* Check all items that are applicable:

- (X) lambda or lambdoid bacteriophages or non-conjugative plasmids are used as vectors.
- () T4 bacteriophages are used as vectors.

As stipulated in Section III-0 of the NIH Guidelines, I agree to comply with the requirement of P1 + Ekl containment for projects using E. coli K-12 host-vector systems. The above information is accurate and complete.



Principal Investigator

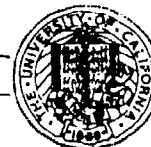
8/1/93

Date

* Pursuant to the biosafety policies of the University of California and NIH, projects involving rDNA that are exempted from the NIH Guidelines or use the E. coli K-12 host-vector system must be registered with the Campus Institutional Biosafety Committee (IBC). All other projects must have a Memorandum of Understanding and Agreement (MOA) filed with the Campus IBC and NIH.

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SANTA BARBARA • SANTA CRUZ

PROFESSOR RAYMOND C. STEVENS
DEPARTMENT OF CHEMISTRY
(510) 643-8285

BERKELEY, CALIFORNIA 94720-1460
E-MAIL: stevens@ncuron1.berkeley.edu
FAX: (510) 643-0883

August 17, 1994

Ms. Patricia McCallister
U. S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland
FAX: 301-619-2937

RE: Contract No. DAMD17-93-C-3118

Dear Ms. McCallister:

I am attaching the second year recertification letters of the emergency response plan approved by the University of California Environmental Health and Safety Office and for the emergency response plan for the subcontract with the University of Wisconsin approved by their Biological Safety Office. I will also send a copy of this material to you today by federal express.

The annual report will follow within a few weeks.

Sincerely yours,

A handwritten signature in black ink, appearing to be "R. Stevens", written over a horizontal line.

Raymond C. Stevens
Assistant Professor of Chemistry

Enc.

UNIVERSITY OF CALIFORNIA, BERKELEY

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OFFICE OF ENVIRONMENT, HEALTH AND SAFETY
UNIVERSITY HALL, 3rd FLOOR

BERKELEY, CALIFORNIA 94720

August 16, 1994

U.S. Army
Medical Research and Development Command
ATTN: SQRD - SF
Fort Detrick
Frederick, MD 21702-5012

RE: Emergency Response Plan for Raymond C. Stevens
College of Chemistry, University of California

Dear Sir/Madam:

The Office of Environment, Health and Safety of the University of California at Berkeley has reviewed the Emergency Response Plan for the laboratory of Professor Raymond C. Stevens, who is under contract with the Army. The nature of Dr. Stevens' research has remained the same and so the Emergency Response Plan has not changed. The City of Berkeley is the first responder for the Campus and there are long-standing cooperative relationships with the City's Office of Toxic Management and the Fire Department, as well as a formal Mitigation Implementation Agreement. If you have any questions about the Emergency Response Plan, please contact Chris Carlson (510-643-6562).

Sincerely,

A handwritten signature in dark ink, appearing to read "Susan L. Spencer".

Susan L. Spencer
Director

SLS/crc

cc: Denise Barndt, Assistant to the City Manager for Emergency & Toxics Mgmt.
Daniel Boggan, Jr., Vice Chancellor - Business and Administrative Services
Chris Carlson, Biosafety Officer
Steven Pedersen, Chair, Safety Committee, College of Chemistry
Raymond C. Stevens, Assistant Professor, College of Chemistry

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OFFICE OF THE CHANCELLOR

BERKELEY, CALIFORNIA 94720

June 16, 1993

Mr. Michael F. Brown, City Manager
Martin Luther King Jr. Civic Center Building
2180 Milvia Street
Berkeley, CA 94704

Dear Mr. Brown:

The University is presently seeking a contract with the U. S. Army to conduct research in furtherance of the Army's Medical Biological Defense Research Program (MBDRP). The University is required by law (Title 10, United States Code, Section 2370) to coordinate annually with the City for the purpose of providing information on the MBDRP and formalizing emergency support. The MBDRP is a research and development program funded by Congress with the mission of providing medical strategies (vaccines, antidotes, treatments) for the defense of military personnel against the use of biological weapons. The work at the University involves the use of Botulinum neurotoxin, an agent which may have the potential to cause disease in humans.

The "Mitigation Implementation Agreement by and between the City of Berkeley and the Regents of the University of California" dated July 26, 1990, is a formal agreement between the University and the City and includes emergency services (fire and police). To facilitate approval of the MBDRP contract, we request documenting our emergency support agreement thorough an endorsement to this letter. The University is taking precautions to minimize the potential for any emergencies; however, in the event of an accident, the following emergency support services may be necessary from the City: fire services, emergency medical transport, emergency spill response, and police assistance. The University Police Department will provide primary police service; the Office of Environment, Health and Safety will provide emergency response assistance, and the Occupational Health Program (University Health Service) will provide emergency medical consultation. Should a case of botulism occur, the antitoxin is readily available through the Centers for Disease Control.

Following is information on Botulinum neurotoxin and the safety precautions implemented by Professor Raymond C. Stevens (College of Chemistry) for the research. Botulinum neurotoxin is a toxin of bacterial origin and is stored as a purified protein solution (seven milligrams per milliliter of solution); the maximum amount of solution employed at any one time is on the order of one milliliter. The primary routes through which disease occurs include: injection or ingestion. Individuals affected by this agent may have nerve paralysis.

Botulinum neurotoxin is a biosafety level two (BSL-2) agent. BSL-2 agents are one of four categories of agents, with BSL-1 being the least hazardous and BSL-4 being the most hazardous. BSL-2 agents include the broad spectrum of indigenous moderate risk agents present in the community and associated with human disease of varying severity. BSL-2 agent can be safely used in activities conducted in an open laboratory area provided the potential for producing aerosols is low. Where aerosols may be generated, the research with the agent is performed in a biological safety cabinet or with other physical containment equipment. Primary hazards to personnel working with BSL-2 agents may include accidental autoinoculation, ingestion, and skin and mucous membrane exposure.

Professor Stevens' laboratory safety program includes the following precautions taken to minimize hazard to personnel, the community, and the environment. All individuals working with the toxin are immunized against the toxin through the Occupational Health Program (University Health Service). The toxin is kept locked up at all times and Professor Stevens controls access. All individuals in the laboratory are instructed about the Standard Operating Procedure and go through a special training program.

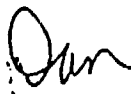
Mr. Brown
June 16, 1993
Page 2

502 Stanley Hall has been assigned for the toxin work. Special equipment to provide both effective personnel protection and emergency support include a dedicated ventilation system with emergency power backup and monitor. The room is kept under negative pressure relative to the building pressure for containment purposes.

We appreciate your acknowledgment of the emergency service support the City provides the University through an endorsement to this letter and your assistance in helping us meet this statutory requirement. This is a continuing annual requirement which must be reported to Congress in September; therefore, for the duration of the contract, each July the University will contact the City and request an acknowledgment of the emergency services provided.

The Office of Environment, Health and Safety (EH&S) has been working with Professor Stevens, if you have questions please contact Susan Spencer (643-8965) or Jack Salazar (643-8676). They will be happy to coordinate a meeting with Professor Stevens.

Sincerely yours,



Daniel Boggan, Jr.
Vice Chancellor - Business and Administrative Services

DB/SLS/jb
Attachment

cc: Denise Johnston, Emergency and Toxics Management, City of Berkeley

Harry Chiladakis, College of Chemistry
Dr. James Seward, Occupational Health Program
Professor Raymond C. Stevens, College of Chemistry
Susan L. Spencer, Environment, Health and Safety

I, William D. Rucker, an official of the City of Berkeley, having the authority to execute this agreement on behalf of the City of Berkeley, do hereby acknowledge its contents and agree to provide the health, fire, and police support identified above.



Signature



Title

Office of Biological Safety
1552 University Avenue
Madison, WI 53705
608/263-2037
FAX 608/262-9059

Biological Safety Committee

12 July 1993

Mr. Philip Vorlander
City of Madison Hazardous Materials Response Unit
325 W. Johnson St.
Madison, WI 53703

Dear Mr. Vorlander:

We are presently seeking a contract with the U.S. Army to conduct research in furtherance of the Army's Medical Biological Defense Research Program (MBDRP). The MBDRP is a research and development program funded by Congress with the mission of providing medical strategies (vaccines, antidotes, treatments) for the defense of military personnel against the use of biological weapons.

Our work involves the use of *Clostridium botulinum* toxin, which is potentially fatal to humans. The investigator involved with this study is Dr. Bibuhti Das Gupta of the Food Research Institute, which is located on this campus at 1925 Willow Drive. Pursuant to Title 10, United States Code, Section 2370 (enclosure 1), we are required by law to coordinate annually with your agency for the purpose of providing information on the MBDRP and formalizing an agreement for emergency support.

Clostridium botulinum toxin is a bacterial toxin. It may be present in the following material located within our laboratory: *in vitro* bacteriological and biochemical materials. The primary routes through which disease occurs include: ingestion; skin, eye and mucous membrane absorption (including the respiratory tract); parenteral inoculation. Individuals affected by this agent may experience the following symptoms and potential adverse effects: neurological symptoms resulting in respiratory failure and death.

Clostridium botulinum toxin is considered to be a biosafety level two (BSL-2) agent. BSL-2 is one of four categories of agents, with BSL-1 being the least hazardous and BSL-4 being the most hazardous. BSL-2 agents include the broad spectrum of indigenous moderate-risk agents present in the community and associated with human diseases of varying severity. These agents can be safely used in activities conducted in an open laboratory area, provided the potential for producing aerosols is low. Where aerosols may be generated, research with these agents is performed in a biological safety cabinet or with other physical containment equipment. Primary hazards to personnel working with BSL-2 agents may include accidental autoinoculation, ingestion and skin and mucous membrane exposure.

When laboratory manipulations of *Clostridium botulinum* toxin involve production of purified toxin, BSL-3 may be indicated. BSL-3 procedures and pertinent facility design features are established for the laboratory in question.

Included among our laboratory safety program are the precautions recommended in the Department of Defense Safety Program (Federal Register 32 CFR Part 627 Friday 10 April 1992). This document describes the precautions taken to minimize hazards to personnel, the community and the environment and is summarized on the attached. In addition to these precautions, the personnel of Dr. Das Gupta's lab are annually examined for their titres against the botulinum toxin and are given booster shots as needed.

Coordination of the following haz-mat emergency responses may be needed from your organization: Fire responders, police and security, emergency first aid, administration of antitoxin, assisted ventilation for respiratory failure.

Special equipment and training to both provide effective emergency support and reduce the potential hazards to emergency personnel include: Identification of agent and use of usual emergency room protective attire.

We request this support from your agency. If the provision of this support is acceptable to your agency, we request documentation of our agreement through an endorsement to this letter. You can endorse our agreement by signing the acknowledgements below. Any questions should be directed to Dr. Joseph Kanabrocki, Office of Biological Safety, 263-2037.

This is a continuing annual requirement that must be reported to Congress in September. Therefore, each July you will be consulted as to the status of this agreement. Our continued agreement, if unchanged, will be verified at that time. Any changes to our agreement will need to be formally executed.

Thank you for your assistance in helping us meet this statutory requirement. Your assistance allows us to perform research that is essential for the development of medical measures that will protect U.S. armed forces throughout the world.

Sincerely,



Robert Erickson, Director
Research Administration-Financial

Enclosures

cc: Dr. B. Das Gupta
Dr. J. Kanabrocki

**Please return endorsement statement
in the first block, next page to:

R. W. Erickson
Rm. 446 Peterson Bldg.
750 University Avenue
Madison, WI 53706

I Official, an official of the Agency, having the authority to execute this agreement on behalf of the Agency, do hereby acknowledge its contents and agree to provide the emergency support identified above, if required.

Frank W. London

Signature

Assistant Fire Chief

Title

29 July 1993

Date

Operations & Training

Position

Annual Review/Recertification

On Date, I consulted with Official, an official of the Agency, who has the authority to execute this agreement. This official has verified that our current agreement remains unchanged.

Joseph Kamalbrock

Signature

Director

Title

17 August 1994

Date

Office of Biological

Position

Safety

Annual Review/Recertification

On Date, I consulted with Official, an official of the Agency, who has the authority to execute this agreement. This official has verified that our current agreement remains unchanged.

Signature

Title

Date

Position